Biochimica et Biophysica Acta, 541 (1978) 535—542

BBA Report

BBA 21477

RAMAN SPECTROSCOPY: A STRUCTURAL PROBE OF GLYCOSAMINOGLYCANS

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(Received January 9th, 1978)

Summary

We report the first Raman spectroscopic study of the glycosaminoglycans chondroitin 4-sulfate, chondroitin 6-sulfate and hyaluronic acid, both in solution and in the solid state. To aid in spectral identification, infrared spectra were also recorded from films of these samples. Vibrational frequencies for important functional groups like the sulfate groups, glycosidic linkages, C-OH and the *N*-acetyl group can be identified from the Raman spectra. Certain differences in the spectra of the different glycosaminoglycans can be interpreted in terms of the geometry of the various substituents, while other differences can be related to differences in chemical composition.

Although Raman spectroscopy has been applied in studying the structure of proteins and nucleic acids, very few applications of this technique have been made to investigate the structure of polysaccharides. The only work to date has been limited to studies of monosaccharides [1—4] and homopolymers of glucose, cellulose [5] and amylose [6, 7]. Very recently Tu et al. [8] have reported the Raman and infrared spectra of hyaluronic acid and its potassium salt in the solid state. These earlier studies show that Raman and infrared spectra are sensitive to (i) the differences between the α and β anomers [1], (ii) differences between different epimers of glucose [4] and (iii) differences between different polymorphic forms of amylose [6, 7].

In this paper, we report the first Raman spectra of a more complex class of polysaccharides, the glycosaminoglycans which form the connective tissue matrix. Our initial experiments focussed on two sulfated glycosaminoglycans (chondroitin 4-sulfate and chondroitin 6-sulfate), and an unsulfated glycosaminoglycan (hyaluronic acid). All of the above glycosaminoglycans are linear polymers having a typical repeating disaccharide unit of the type $[A\text{-}B]_n$,

where A is glucuronic acid and B is either *N*-acetylgalactosamine (chondroitin 4-sulfate and chondroitin 6-sulfate) or *N*-acetylglucosamine (hyaluronic acid) [9]. To aid in the identification of spectral lines we have also measured the infrared spectra of films cast from the same samples as used in the Raman studies. Infrared spectra of glycosaminoglycans have been reported previously [8, 10—12]; however, at the time of these earlier studies, the characterization of the glycosaminoglycans was not as advanced as it is today, and thus we felt it desirable to obtain infrared data on the present samples which are very well characterized.

Our results demonstrate that there are significant differences in the Raman spectra of the sulfated and unsulfated glycosaminoglycans and, furthermore, that the Raman spectra are sensitive to the orientation of the sulfate group relative to the pyranose ring. Certain differences in the spectra of the different glycosaminoglycans can be related to differences in chemical composition, while other differences are interpretable in terms of the geometry of various substituents. We propose assignments for vibrational frequencies of several groups. In particular, the identification of the vibrations of the sulfate groups and the glycosidic linkages may be of potential significance in determining the conformations of the glycosaminoglycans in highly hydrated states, and in understanding the possible role of the sulfate groups in the interactions between collagen and the sulfated glycosaminoglycans.

The Raman spectra were taken with the 5145-Å line of a Spectra Physics model 164-03 Ar^+ ion laser and Spex Ramalog IV double grating monochromator system. The spectra were recorded with 5 cm^{-1} resolution and incident power levels of 40—100 mW using either a scanning speed of 0.1 cm^{-1}/s or a computerized signal averaging procedure (Nicolet 1180 Data Collecting and Signal Averaging Package). With the latter method the spectrum is recorded by a fast scan (typically 3 cm^{-1}/s) and many such scans (typically 100) are signal averaged. Since the data are stored digitally it is possible to eliminate high frequency noise and subtract the background with Fourier transform methods. In performing these manipulations of the data, care is taken not to introduce any additional peaks or change the frequencies of the Raman lines.

Reference standard preparations of chondroitin 4-sulfate and chondroitin 6-sulfate were obtained from Dr. M.B. Mathews, University of Chicago. Many details of isolation procedures and characterization methods are described elsewhere [13]. Purified chondroitin 6-sulfate from shark cartilage was also obtained from Calbiochem, Monsey, New York. Purified hyaluronic acid from umbilical cord was obtained from Dr. D.A. Swann, Harvard Medical School [14]. The monosaccharides D-glucuronic acid, *N*-acetylglucosamine, *N*-acetylgalactosamine, glucose, galactose and glucose 6-sulfate were obtained from Sigma Chemicals, St. Louis, Mo. and were used without further purification.

Fig. 1 shows the Raman spectra of 5% aqueous solutions of sodium hyaluronate, chondroitin 6-sulfate and chondroitin 4-sulfate for the region 800—1500 cm^{-1}. Fig. 2 shows the Raman spectra of powders of chondroitin 4-sulfate and chondroitin 6-sulfate for the region 300—1800 cm^{-1}. The raw data for chondroitin 6-sulfate and the Fourier transformed spectrum with background eliminated are both shown in Fig. 2 for the purposes of illustrating

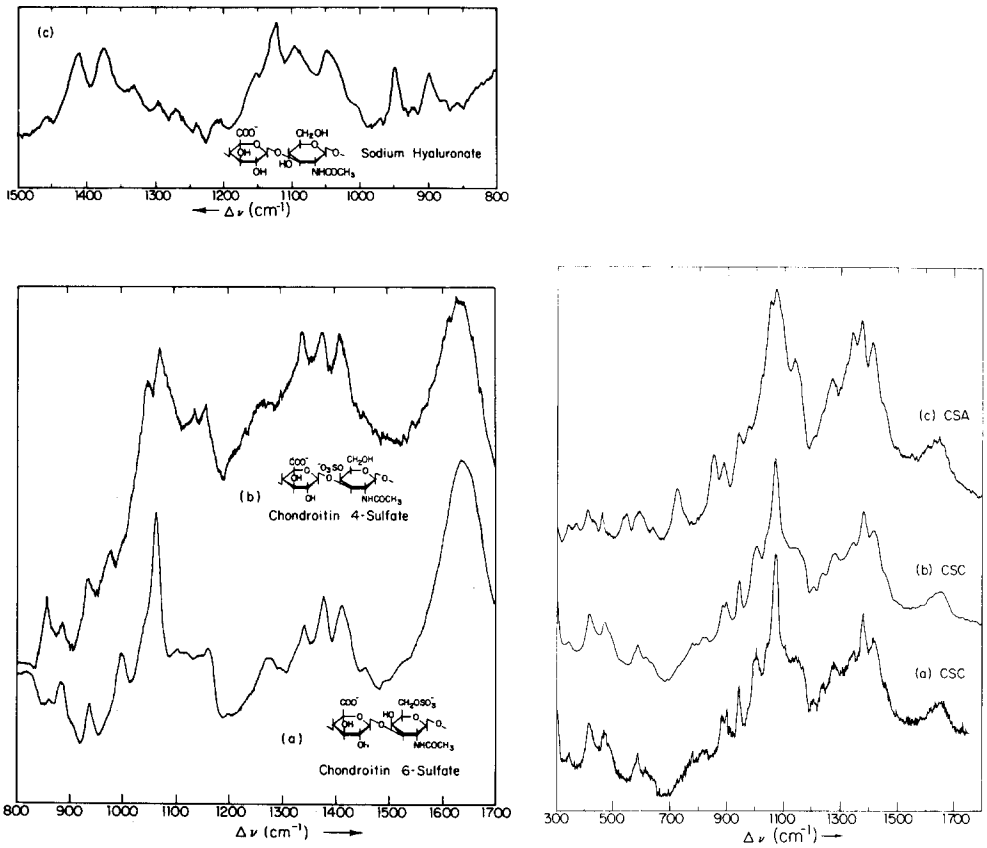


Fig. 1. Raman spectra of 5% solutions of glycosaminoglycans in water: a, chondroitin 6-sulfate; b, chondroitin 4-sulfate; and c, sodium hyaluronate. The incident wavelength is 5145 Å, power 100 mW and resolution 5 cm^{-1}. The spectra shown in a and b were recorded with the Signal averaging package and show 100 scans averaged and Fourier transformed to reduce noise. The spectrum shown in c was recorded with the single scan mode of the Spex compudrive at a scan rate of 0.1 cm^{-1}/s.

Fig. 2. Raman spectra of powders of chondroitin 6-sulfate (a, b) and chondroitin 4-sulfate (c). Incident laser wavelength = 5145 Å, power 20 mW, spectral resolutions, 5 cm^{-1}. The spectrum shown in a is the raw data signal averaged for 30 scans, whereas that shown in b is Fourier transformed to remove the high-frequency noise. A comparison of the spectra in a and b shows that data manipulation does not introduce any artifacts into the spectrum: it only reduces the noise, and this makes the weaker peaks show up more clearly. The spectrum shown in c is the average of 100 scans and has been Fourier transformed. CSA, chondroitin 4-sulfate, CSC, chondroitin 6-sulfate.

the fact that computer manipulation of data does not introduce any artifacts into the spectrum. The spectra in the solid state are essentially the same as those of solutions, with somewhat better resolution of the low frequency bands. This is not surprising in view of the fact that the solid samples were in the form of an amorphous powder of a highly polymeric substance and thus there is no net orientation of any group in the polymeric molecule. Fig. 3 shows the infrared spectra of films cast from chondroitin 6-sulfate, chondroitin 4-sulfate and hyaluronic acid. The infrared spectra for the high frequency region (i.e., 2500—3500 cm^{-1}) are also shown in Fig. 3.

Most of the peaks in the Raman spectrum can be associated with vibrations of D-glucuronic acid and *N*-acetyl-D-glucosamine or *N*-acetyl-D-galactosamine as shown in Table I. Assignments were made by comparing the spectra of the

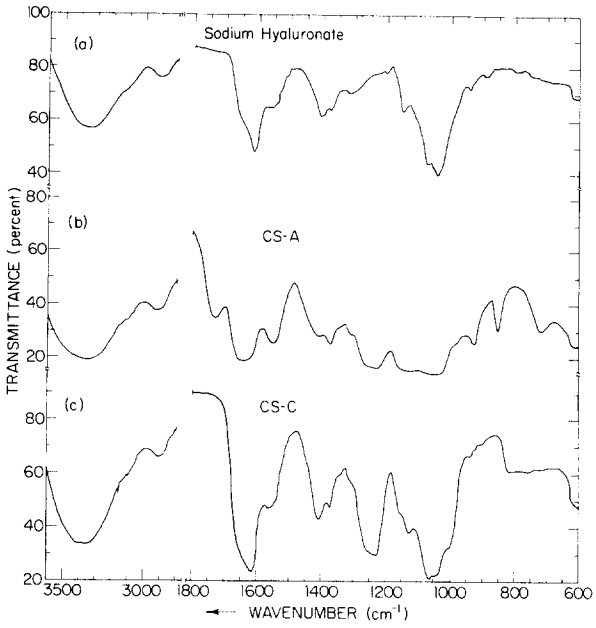


Fig. 3. Infrared spectra of films cast from (a) sodium hyaluronate, (b) chondroitin 4-sulfate and (c) chondroitin 6-sulfate.

polysaccharides with the relevant monosaccharides. Details of some of the vibrations are discussed below.

Various suggestions have been made concerning the role of the sulfate groups in the interaction between sulfated glycosaminoglycans and collagen [15]. One possible way of probing these interactions could be to monitor the vibrational frequencies characteristic of the sulfate groups in the glycosaminoglycans.

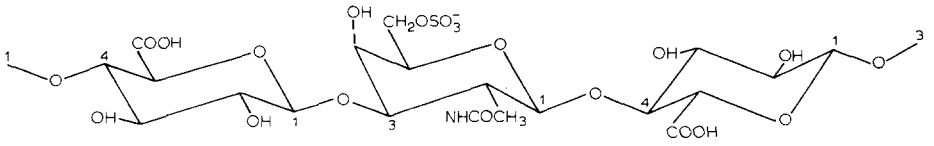
The strongest peaks in the Raman spectra of chondroitin 6-sulfate and chondroitin 4-sulfate at 1064 and 1079 cm^{-1}, respectively, can be identified as the symmetric stretching vibration of the OSO_3^- group. This assignment is consistent with (i) the absence of this peak in hyaluronic acid which does not have any sulfate groups; (ii) the occurrence of a strong peak at 1064 cm^{-1} in the Raman spectrum of glucose 6-sulfate (Bansil, R., unpublished) which is not present in glucose, and (iii) the identification of the doublet at 1064 and 1080 cm^{-1} as the OSO_3^- symmetric stretch in crystalline sodium ethyl sulfate [16]. This vibration is weak in the infrared, whereas the asymmetric OSO_3^- stretching vibration at 1237 cm^{-1} (1232 cm^{-1} in chondroitin 4-sulfate) is strong in the infrared and weak in the Raman. The latter vibration is part of the broad band at 1271 and 1269 cm^{-1} in the solution spectra of chondroitin 6-sulfate and chondroitin 4-sulfate, respectively (see Fig. 1); it can be resolved in the solid-state spectra (see Fig. 2). The slight difference in the frequency of the OSO_3^- stretching vibration in chondroitin 4-sulfate and chondroitin 6-sulfate is probably due to the different environment of the sulfate groups in these two glycosaminoglycans. As shown in Fig. 4, the sulfate group would be equatorial in chondroitin 6-sulfate and axial in chondroitin 4-sulfate, if the *N*-acetylgalactosamine residue were in the C 1 chair conformation.

TABLE I

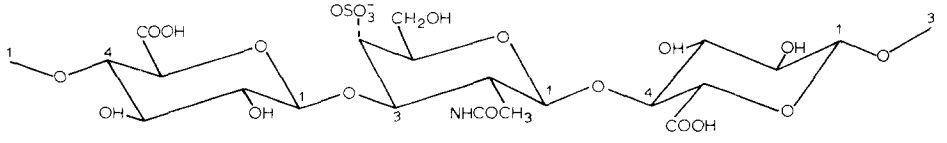
RAMAN FREQUENCIES OF GLYCOSAMINOGLYCANS AND MONOSACCHARIDES

Chondroitin 6-sulfate	Chondroitin 4-sulfate	Sodium hyaluronate	D-Glucuronic acid	*N*-Acetyl-galactosamine	*N*-Acetyl-glucosamine	Assignment
			1727 (s)			C=O vibration of COOH [18]
1615 (s) 1640 (sh)	1635 (s)	1620 (s) (1640 sh)		1632 (s)	1631 (s)	Amide I
1560 (m)	1550 (m)	1565 (m)		1590 (w)	1550 (w)	Amide II Shows mainly in IR
1459 (w,br)	1456 (w,br)	1458 (w,br)		1467 (m)	1462 (m)	CH_2 deformation [18]
1413 (s)	1411 (s)	1412 (s)				COO^- symmetric [18]
1377 (s)	1376 (s)	1375 (s)		1381 (s)	1383 (s)	CH_3 symmetric deformation [18]
			1363 (s,br)			
1340 (s)	1341 (s)	1331 (m)		1331 (s)	1327 (s)	Amide III [17]
1320 (sh)	1314 (sh)					
1298 (w)						
	1277 (w)					
1271 (m,br)	1269 (m,br)	1268 (m)	1273 (w)	1275 (s)	1266 (w)	
1237 (w)	1232 (w)		1232 (sh)			SO_3^- asymmetric stretch [10, 11, 16] (strong in IR)
1206 (w)	1210 (w)	1206 (m)	1205 (w)		1206 (sh)	
1159 (m)	1157 (w)	1153 (m)	1155 (m)	1148 (sh)	1156 (sh)	
1120 (w)	1137 (w)	1124 (s)	1120 (vs)		1128 (vs)	$C_{(4)}$-OH, C-H and C-OH deformation [1, 2]
1100 (w)	1089 (sh)	1096 (m)		1089 (s)	1087 (m)	C-OH [1, 2]
1062 (vs)	1079 (s)					SO_3^- symmetric stretch
1050 (sh)	1050 (s)	1050 (m)	1059 (s)	1052 (sh)	1055 (s)	Partly C-OH
1035 (sh)	1035 (sh)		1038 (w)	1023 (sh)		
1004 (sh)	1004 (sh)	1004 (sh)			998 (w)	
995 (s)	978 (m)					C-O-(S)
975 (w,sh)	961 (sh)	970 (w)		970 (s)	964 (s)	
		960 (sh)				
937 (s)	937 (ms)	949 (s)	941 (sh)			Skeletal C-O-C linkage vibration
		922 (w)	915 (w)		917	
903 (sh)					902	
884 (s)	891 (m)	899 (s)	864 (m)	877 (m)		$C_{(1)}$-H deformation for β anomers [1, 2, 4]
			847 (m)	825 (m)		$C_{(1)}$-H deformation for α anomer [1, 2, 4]
820 (m)	853 (ms)					C-O-S (strong in IR) [10, 11]
780 (w)	758 (sh)		771 (m)		781 (w)	
	725 (m)	708	707 (vw)	716		
637 (w)	642 (w)	676 (w)	627 (w)	623 (m)		
578 (w)	594, 547 (m)	540	572 (m)	595, 527 (m)	547, 525 (m)	
483 (sh)	491 (w)	490	537	486 (w)	458 (m)	
459 (w)	462 (m)	474	458 (s)			Mainly
437 (sh)	439 (sh)	443		442 (m)		skeletal
414 (sh)	412 (m)	413	417 (m)		409 (m)	modes
381 (s)	375 (w)		397 (w)	375 (w)		
342 (w)	342 (w)	342 (w)	344 (w)			

(s), strong; (m), medium; (w), weak; (sh), shoulder; (br) broad; (v), very; IR, infrared.



(a) Chondroitin 6-sulfate



(b) Chondroitin 4-sulfate

Fig. 4. A schematic diagram showing the configuration of (a) chondroitin 6-sulfate, (b) chondroitin 4-sulfate, where the *N*-acetyl-D-galactosamine and D-glucuronic acid residues are in the C1 chair conformation, and both the linkages are equatorial. For purposes of clarity all equatorial bonds are shown as full lines and all axial bonds are shown as dotted lines. Note that this configuration places the sulfate group in chondroitin 6-sulfate in the equatorial configuration and that in chondroitin 4-sulfate in the axial configuration.

By comparing the spectra of the chondroitin sulfates with that of hyaluronate other vibrations of the sulfate group can be identified at 995 and 820 cm^{-1} in chondroitin 6-sulfate and 977 and 853 cm^{-1} in chondroitin 4-sulfate. The vibrations at 820 cm^{-1} in chondroitin 6-sulfate and 853 cm^{-1} in chondroitin 4-sulfate have been identified as the asymmetric vibration of the C-O-S linkages on the basis of infrared studies [10, 11]. The difference of approx. 35 cm^{-1} in chondroitin 4-sulfate and chondroitin 6-sulfate has been interpreted to reflect the equatorial configuration of the OSO_3^- group in chondroitin 6-sulfate and the axial configuration in chondroitin 4-sulfate [11] (see Fig. 4). The 820 cm^{-1} band in the Raman and infrared spectra of chondroitin 6-sulfate is not as clearly resolved as the 853 cm^{-1} band of chondroitin 4-sulfate.

We propose that the 995 cm^{-1} band in chondroitin 6-sulfate and the 977 cm^{-1} band in chondroitin 4-sulfate correspond to the symmetric vibration of the C-O-(S) linkage, reflecting the effect of the sulfate group on the C-O link. This vibration is much stronger in the Raman than in the infrared spectrum. The difference in the frequency of this band in chondroitin 4-sulfate and chondroitin 6-sulfate is again related to the axial vs. equatorial arrangement of the sulfate group. The 995 cm^{-1} band in chondroitin 6-sulfate corresponds to the 1011 cm^{-1} band of glucose 6-sulfate, at which frequency the spectrum of glucose shows only a shoulder ($I_{1011}/I_{1462}(CH_2) \approx 1$ in glucose and $I_{1011}/I_{1462} \approx 3$ in glucose 6-sulfate) (Bansil, R., unpublished).

Deformation modes of the sulfate group lie in the region below 600 cm^{-1}. However, we have not attempted to identify these vibrations because the torsional and skeletal modes of the pyranose ring also lie in this region. The skeletal modes of the pyranose ring are extremely sensitive to changes in the substituents at various positions, and therefore one needs to first study this region for the unsulfated monosaccharides in detail before identifying the deformation modes of the sulfate group.

From an analysis of Raman data for α and β glucose in the crystalline state [1, 2], and from an analysis of infrared data on a number of monosaccharides [4], it has been proposed that a $C_{(1)}$-H deformation mode occurs at 847 cm^{-1} if the hydrogen at position 1 is equatorial (as in α-glucose) and at 898 cm^{-1} if the hydrogen at position 1 is axial (as in β-glucose). In the Raman spectra of the three glycosaminoglycans examined in this study we consistently found only the higher frequency vibration (884 cm^{-1} in chondroitin 6-sulfate, 889 cm^{-1} in chondroitin 4-sulfate and at 899 cm^{-1} in hyaluronic acid). This shows that the hydrogen at position 1 in all of these glycosaminoglycans is axial. Since $C_{(1)}$ is involved in both glycosidic linkages ($1\rightarrow4$ and $1\rightarrow3$, see Figs. 1 and 4) this finding is in agreement with the generally accepted view that the linkages in chondroitin 4-sulfate, chondroitin 6-sulfate and hyaluronic acid are of the β type [9]. Tu et al. [8] draw a similar conclusion regarding the correlation of the 847 cm^{-1}/898 cm^{-1} bands and the configuration at $C_{(1)}$ from their Raman and infrared studies of hyaluronic acid in the solid state. The small differences between the frequency of this band in the different glycosaminoglycans may be due to conformational differences. Further studies of this frequency as a function of factors which affect the conformation of the glycosaminoglycans are needed to validate this suggestion.

The vibration at 937 cm^{-1} in chondroitin 6-sulfate (941 cm^{-1} in chondroitin 4-sulfate and 949 cm^{-1} in hyaluronic acid) appears to be a skeletal vibration of the C-O-C linkages. This is based on the observation that neither glucuronic acid nor *N*-acetylgalactosamine/*N*-acetylglucosamine has a vibration at this frequency. The closest vibration of *N*-acetylgalactosamine is at 970 cm^{-1} (964 cm^{-1} in *N*-acetylglucosamine), which is seen as a shoulder in the glycosaminoglycans spectra. Thus it is unlikely that the 936 cm^{-1} vibration corresponds to the 970 cm^{-1} vibration of the *N*-acetyl group. Koenig et al. [6, 7] have observed a vibration at 946 cm^{-1} in V-amylose (936 cm^{-1} in B-amylose) which they assign as a skeletal mode involving the cooperative vibration of the glycosidic oxygen atom and the ring oxygen atoms. Since amylose has $\alpha(1\rightarrow4)$ linkages whereas the glycosaminoglycans have $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ linkages, it appears that this vibration depends little on whether the linkage is axial (α) or equatorial (β).

Another vibration which is a useful marker for the determination of the structure of glycosaminoglycans is the 1130 cm^{-1} band in hyaluronate. This strong band is always present in glucose derivatives but is absent in galactose derivatives. This vibration has been assigned to a C-H and C-OH deformation mode on the basis of deuterium exchange studies [1, 2]. We suggest that because of its absence in galactose this mode is largely a $C_{(4)}$-H, $C_{(4)}$-OH deformation and occurs only when the OH at position 4 is equatorial.

Due to the presence of the sulfate vibrations in the chondroitin sulfates, C-OH deformation modes which occur in the 1000—1200 cm^{-1} range are masked. However, these frequencies show up clearly in sodium hyaluronate. By comparing the Raman spectra of sodium hyaluronate in H_2O and ^2H_2O, we assign the lines at 1096 and 1124 cm^{-1} as largely due to C-OH vibrations. In addition the line at 1050 has some C-OH contribution, because its intensity decreases on deuterium exchange.

In the Raman spectrum of aqueous solutions only the amide III band

shows up at 1331 cm^{-1}, characteristic of the *cis* arrangement of the C=O and N-H groups with respect to the C-N bond [17]. However, in the infrared spectrum of chondroitin 6-sulfate (Fig. 3) we can see the amide I band at 1610 cm^{-1} with a shoulder at 1650 cm^{-1} and the amide II band at 1560 cm^{-1} The CH_3 symmetric deformation frequency occurs as a strong peak at 1373 cm^{-1} in all glycosaminoglycans, whereas the CH_2 deformation at approx. 1452 is very broad and weak as compared to the monosaccharides. The strong band at 1411 cm^{-1} is due to the symmetrical vibration of the COO^- group of the glucuronate residue [18].

In summary, we find that the Raman spectra of glycosaminoglycans contain a great deal of detailed information about the structure of these molecules. Several bands such as the sulfate vibration, the vibration of the hydrogen atoms at position 1 and the 1130 cm^{-1} vibration characteristic of glucose derivatives can be used as 'finger prints' for the presence of these groups and their spatial arrangement relative to the pyranose ring. Moreover, we have identified certain frequencies, such as the vibration of the hydrogen atoms at position 1 and the C-O-C linkage skeletal vibration, which may be of potential use in studying the conformations of glycosaminoglycans in the hydrated state. The identification of the sulfate vibrations may be of importance in studying the interactions of glycosaminoglycans with collagen, since there have been suggestions that the sulfate groups are involved in collagen-glycosaminoglycan interactions. Preliminary work along these lines is underway.

We thank Professor M.B. Mathews, University of Chicago, for providing us with purified samples of chondroitin sulfates A and C, and Professor D.A. Swann, Harvard Medical School and Shriners Burns Institute at Massachusetts General Hospital for providing the sodium hyaluronate used in this study. We acknowledge helpful discussions with Professor R.C. Lord and thank Mr. William Huffman for technical assistance.

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